ASPECTS OF GROWTH AND NITROGENASE ACTIVITY OF THE PHOTOSYNTHETIC CYANOBACTERIUM *NOSTOC MUSCORUM* IN CONTINUOUS CULTURE.

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ABSTRACT — The level of photosynthetic oxygen evolution, chlorophyll, protein, nitrogenase and nitrate reductase activities were studied in the cyanobacterium *Nostoc muscorum* growing in continuous culture under various nitrogen sources (N_2-free medium, NO_3^- or NH_4^+-media). The growth and production rate of *N. muscorum* were optimal in the presence of NO_3^- rather than in the presence of NH_4^+ or molecular N_2. The rate of photosynthetic O_2 evolution showed correlation with the increased chlorophyll and protein level in NO_3^--grown cultures. The level of the key enzyme of nitrogen metabolism in cyanobacteria (evaluated by nitrogenase and nitrate reductase activities) were maxima in NO_3^-- cultures, while NH_4^+-grown cultures had the lowest level.

RÉSUMÉ — L'évolution de la production d'oxygène photosynthétique, les teneurs en chlorophylle et en protéine, ainsi que l'activité de la nitrogénase et de la nitrate réductase, ont été étudiées chez la cyanobactérie *Nostoc muscorum* maintenue en culture continue, en présence de sources d'azote variées (azote gazeux N_2, nitrateNO_3^- ou ammonium NH_4^+). La croissance et le taux de production de *N. muscorum* ont été supérieurs en présence de nitrate NO_3^-, qu'en présence d'une source d'azote ammoniacal NH_4^+ ou moléculaire N_2. L'évolution de la production d'oxygène photosynthétique présente une corrélation avec l'augmentation des teneurs en chlorophylle et en protéine, dans les cultures où l'azote est fourni sous forme de nitrate. La quantité d'enzyme-clef du métabolisme azoté chez les cyanobactéries (évaluée par la mesure des activités de la nitrogenase et de la nitrate reductase) a été maximale dans les cultures où l'azote est présent sous forme de nitrate, tandis qu'elle a été minimale en présence d'azote ammoniacal. (Traduit par la rédaction).

KEY WORDS : *Nostoc muscorum*, growth, production rate, nitrogenase activity.

INTRODUCTION

Cyanobacteria play a major role among the micro-organisms which reduce dinitrogen to ammonia, and a range of cyanobacterial species known to be N_2-fixing exist in rice field ecosystems. Virtually all the dominant cyanobacteria in rice fields

Source : MNHN, Paris

are N_2-fixing, and therein lies the explanation of how rice, which provides the staple diet for about one-half of the world's population, has been grown continuously in paddy soils for many centuries without addition of fertilizer (Roger & Watanabe, 1984; Watanabe *et al.*, 1987; Abd-Alla *et al.*, 1994). Various factors affect the growth of cyanobacteria in paddy fields including physical, biological and soil factors. Cyanobacteria can be cultured either in laboratory enclosed bioreactors or in open pond systems utilizing sunlight (Borowitzka & Borowitzka, 1989; Tapie & Bernard, 1989).

Continuous culture technique enable to use very low nutrient concentrations and to obtain a dynamic equilibrium between the nutrient input and algal growth (Müller, 1972). A desired cell density can be maintained either by controlling the levels of the limiting nutrients in the reservoir or by controlling the rate of their inflow. In this well defined environment, the desired growth can be easily selected and maintened for a long period at any rate between zero and maximum.

In the present study, we have investigated some aspects of growth and enzymatic activities of the photosynthetic cyanobacterium *Nostoc muscorum* with respect to nitrogen sources using a continuous culture system.

MATERIALS AND METHODS

Culture conditions

The clonal axenic culture of *Nostoc muscorum* Agardh was grown photoautotrophically in modified Chu No. 10 medium (Gerloff *et al.*, 1950) under continuous light (50 $\mu E\ m^{-2}s^{-1}$) at 28 $\pm$ 1°C using chemostat culture at a constant dilution rate of 0.25 day^{-1} as described by Müller (1972). The N_2-medium represents combined nitrogen free medium, while media containing 5 mM $NaNO_3$ or 1 mM NH_4Cl have been referred to as NO_3^- or NH_4^+-media, respectively. The pH of the medium was maintained at 8.2 $\pm$ 0.1 by buffering with HEPES-NaOH buffer. There was no change in the pH of the medium during the experiments period. The air supply to the N-limited chemostats was passed through 1 N H_2SO_4 to remove ammonia before bathing through water. Autoclaved medium in 10 l reservoirs was continuously pumped into the cultures using Micro-pump (MP/K_2).

Growth measurements

The absorbance at 665 nm was measured to monitor changes in the culture biomass (Singh *et al.*, 1978). When the absorbance did not change more than 5 % over 5 days, the cultures were considered to be in steady-state. At that time, the entire culture volumes were sampled for composition and metabolic measurements. Cell number was counted in 1 ml medium obtained after cell swirling. Chlorophyll *a* was determined colorimetrically after acetone extracted as recommended by Metzner *et al.* (1965).

Source : MNHN, Paris

Estimation of O_2-evolution

At steady-state, photosynthetic O_2-evolution was measured polarographically at 30°C in 3 ml buffer by illuminating the cyanobacterial suspension with saturating light intensity at 87.5 $\mu Em^{-2}s^{-1}$ using a Clark type oxigen electrode (Rank) as described previously (Peschek & Schmetterer, 1978; Sharma *et al.*, 1979).

Nitrogenase activity

The acetylene reduction technique of Stewart *et al.* (1971) was used to assay the enzyme activity.

Nitrate reductase activity

This was assayed colorimetrically in the cell-free aqueous extract by diazotation of nitrite formed using the method of Harper (1972).

Analytical methods

Nitrite was estimated by diazocoupling reaction (Snell & Snell, 1949); cellular protein was estimated after trating the cells with 10 % trichloroacetic acid (Lowry *et al.*, 1951).

RESULTS AND DISCUSSION

The growth of *Nostoc muscorum* was studied in modified Chu No. 10 media either in the presence of a fixed nitrogen source such as NO_3^- or NH_4^+ or in the absence of an exogenous fixed nitrogen source. The continuous culture technique was used at a constant dilution rate of 0.25 day^{-1} in all experiments. At the steady-state when growth rate (μ) equals dilution rate (D) the growth parameters revealed significant differences with respect to the nitrogen source in the inflow medium (Table I). The NO_3^--grown culture, accumulated the maximum amount of chlorophyll *a* and protein in addition to cell number. The chlorophyll and protein levels under this condition increased more than twofold over in culture free nitrogen. Moreover, the NO_3^--grown cultures had a significantly higher level of chlorophyll *a* and protein when compared to NH_4^+-grown cultures. The poor growth in NH_4^+-medium has been attributed to the severe drop in pH of the external medium, following rapid NH_4^+ uptake and accumulation within the cell (Fogg *et al.*, 1973).

The production rate refers to the biomass which overflows the culture vessel per time unit. Concerning the cell number, chlorophyll *a* and total protein contents, the rate of production was higher in culture containing NO_3^- than in NH_4^+ or N_2-free media, while the rate of dry weight increased in the N_4 medium more than in NO_3^- or N_2-free media. It is suggested that cell number and chlorophyll were the best measurements of growth and production rate of cyanobacterium.

Source : MNHN, Paris

Table I. effect of nitrogen source in inflow medium on the growth and production rate of *N. muscorum* at a constant dilution rate (0.25 day^{-1}).

Parameters	Nitrogen sources		
	N_2	$NaNO_3$ (5 mM)	NH_4Cl (1 mM)
Cell number (10^8 l^{-1})	10.3 ± 0.1	11.6 ± 0.7	5.8 ± 0.3
Dry weight (mg l^{-1})	53.0 ± 6.1	64.0 ± 5.1	71.0 ± 7.2
Chlorophyll *a* (μg l^{-1})	6.7 ± 0.1	12.7 ± 0.07	5.3 ± 0.09
Protein (mg l^{-1})	6.1 ± 0.3	13.6 ± 0.7	9.3 ± 0.8
(Production rate)			
mg dry weight l^{-1} day^{-1}	10.6 ± 0.7	12.8 ± 0.7	14.2 ± 1.1
Cell number 10^8 l^{-1} day^{-1}	2.06 ± 0.1	2.32 ± 0.3	1.16 ± 0.1
μg chlorophyll *a* l^{-1} day^{-1}	1.34 ± 0.1	1.94 ± 0.2	1.06 ± 0.1
mg protein l^{-1} day^{-1}	1.22 ± 0.3	2.32 ± 0.7	1.86 ± 0.2

Each value represent the mean of three replicates ± standard error.

The rate of phototsynthetic O_2 evolution, of *Nostoc muscorum* grown in chemostat culture, also appeared correlated with the increased chlorophyll and protein level in NO_3^--grown cultures. In NO_3^--grown culture, it was more than twice as high compared to N_2- or NH_4^+-grown cultures, when expressed either on cell basis or chlorophyll basis (Table II). Inhibition of photosynthesis is the prime reason for the observed low growth in NH_4^+-medium, by imposing a limitation on ATP availability (Bagghi *et al.*, 1985, Fernandez-Valiente *et al.*, 1991).

The failure of N_2 not being a good source of nitrogen as NO_3^- is because under the aerobic growth conditions, the N_2 assimilatory enzyme nitrogenase, remains confined only to heterocystes (Stewart, 1973; 1980; Singh *et al.*, 1978; Bagghi *et al.*, 1985). The level of the key ensyme of nitrogen (N_2) metabolism in cyanobacteria (as tested by nitrogenase activity) was maximum in NO_3-grown culture, while NH_4^+-grown culture had the lowest nitrogenase level (Table II). In this respect, Guerrero & Larra (1987) stated that the addition of ammonia to the cyanobacterial culture repressed the nitrogenase activity even at low concentrations. Since ammonia or its metabolites repress the formation of heterocysts, it has been difficult to establish wether inhibition of N_2 fixation is due to an inhibition of heterocyst differentiation or to a repression of nitrogenase synthesis. Furthermore, ammonia may inhibit nitrogenase activity *in vivo* by interrupting ATP or reductant supply (Rowell & Kerby, 1991).

Similarly, the level of nitrate reductase activity in NO_3^--grown culture was significantly higher than in N_2-culture when expressed on cell number basis. The low level of nitrate reductase in NH_4^+-grown culture is however due to repression of nitrate reductase by NH_4^+ (Bagghi *et al.*, 1985). On the other hand, expressing nitrate reductase activity on the basis of total cellular protein reversed the pattern for enzyme activity in N_2 or NO_3^--grown cultures. Generally in N_2-grown cultures, N_2-fixation is confined only to heterocysts, the limited availability of fixed N_2 results

Source : MNHN, Paris

Table II. Rate of photosynthetic O_2 evolution, nitrogenase and nitrate reductase activities in the cyanobacterium *N. muscorum* grown in chemostat culture under different nitrogen sources at constant rate (0.25 day^{-1}).

Nitrogen source	Photosynthetic O_2 evolution		Nitrogenase activity		Nitrate reductase activity	
	Cell basis μ mol O_2/h/10^8 cells	Chlorophyll basis μ mol O_2/h/ mg Chl.*a*	Cell basis n mol C_2H_2/h/10^8 cells	protein basis n mol C_2H_2/mg protein	Cell basis μg NO_2/h/10^8 cells	Protein basis μ NO_2-N/h/mg protein
N_2	7.11 ± 0.3	165.1 ± 7.3	8.5 ± 0.1	2.8 ± 0.3	3.1 ± 0.1	3.9 ± 0.3
$NaNO_3$ (5 mM)	15.80 ± 0.9	361.2 ± 9.7	12.6 ± 0.3	3.7 ± 0.1	5.6 ± 0.3	1.9 ± 0.1
NH_4Cl (1 mM)	6.9 ± 0.7	187.9 ± 6.3	5.4 ± 0.2	0.81 ± 0.1	1.1 ± 0.0	0.9 ± 0.3

Each value represent of thre mean of three determinations ± standard error.

Source : MNHN, Paris

in non accumulation of protein and pigments. On the other hand, NO_3^- can be reduced in all cells of filament by nitrate reductase via a process which is directly coupled to photolysis of water and occurs even in the absence of CO_2 (Flores *et al.*, 1983). In view of nitrogen-source-dependent variations in pigment and protein level in *Nostoc muscorum* cells, it is suggested that the cell number is a better parameter to express growth and enzymatic activities rather, than the pigment level.

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Source : MNHN, Paris

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